THE DIRECT DETERMINATION OF BOUND GLUTARALDEHYDE IN GLUTARALDEHYDE-TANNED COLLAGEN*

ABSTRACT

A direct method for assessing bound glutaraldehyde in leathers tanned with this aldehyde was developed. The method is based on the finding that hyrolyzates of this leather showed a characteristic absorption maximum at 265 mμ. Miniature tanning experiments on a quantitative basis were conducted, and absorbance at 265 mμ was correlated with uptake of glutaraldehyde.

INTRODUCTION

The tanning properties of aldehydes have been the subject of numerous investigations, especially with reference to formaldehyde tanning (1–5). In recent years, new impetus was given to research in the field of aldehyde tanning by the availability of some new aldehydes, particularly dialdehydes (6–8). As a result of this renewed interest, the versatile tanning properties of glutaraldehyde were established (9–13). It was demonstrated that the glutaraldehyde tannage was rapid and efficient (9); that it can be accomplished over a wide pH range (7, 9); that it can be used in combination with conventional tanning agents, such as chrome (11); and that glutaraldehyde imparts unusual properties to leather, such as resistance to perspiration and resistance to hot, soapy water (10). Glutaraldehyde has, in fact, become established as a new tanning agent that is used commercially to some extent in tanning of leather, principally in combination with chrome (13).

While information on the tanning properties of glutaraldehyde was important and thoroughly studied, data on its fixation were rather meager and unreliable. This was so primarily because a direct method for estimating bound glutaraldehyde did not exist. Previous investigators estimated fixation by an indirect means assuming fixed aldehyde to be the difference between 100 and the sum of hide substance, fat (solvent extractable), and ash, on a moisture-free basis (6, 10).

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In a different approach, but still an indirect estimation, data on fixation were based on analysis of the tanning liquor, assuming that aldehyde lost from solution was bound to the hide substance (9). Such indirect methods are inaccurate, non-specific and unreliable and leave much to be desired. In this paper, we report a new method for the direct determination of bound glutaraldehyde in leather using a spectrophotometric method of analysis (14).

EXPERIMENTAL

Quantitative Tanning Procedure.—To permit a material balance for glutaraldehyde in the liquid and skin phases of the tanning system, miniature tanning experiments were conducted applying analytical techniques. The procedure was as follows:

Specimens (5.0 g.) of pickled degreased cabretta, or chrome-tanned cabretta in the blue, obtained from a tannery, were weighed into tared 125 ml. Erlenmeyer flasks, and 0.5 g. of NaCl and five ml. of a standardized glutaraldehyde solution of known concentration were added. The desired pH was maintained by the addition of 0.2 g. of the appropriate buffering agent. The total weight of skin plus aqueous phase was determined and the flask stoppered and shaken mechanically for two hours at room temperature. At the end of this period, the tanned cabretta skin was removed and wrung by squeezing, combining the expressed liquor with the spent tanning liquor in the Erlenmeyer. The tanning liquor was analyzed for residual glutaraldehyde content, as described below.

Immediately after wringing, the glutaraldehyde-tanned cabretta skin was freed of uncombined glutaraldehyde by washing overnight in running tap water. The washed leather was then blotted and dried to constant weight at 70°C. The weight of dry leather was subtracted from the total weight of the tanning system to obtain the actual weight of the tanning liquor or the aqueous phase of the tanning system. The weight of the tanning liquor, together with its glutaraldehyde content, was used to calculate the total unreacted glutaraldehyde in the spent tanning liquor. The difference between the initial and final amount of glutaraldehyde in the aqueous phase was assumed to be the quantity of glutaraldehyde taken up by the skin. The data obtained are summarized in Tables I, II and III.

Analysis for Glutaraldehyde in Tanning Liquor.—The analysis was based on formation of the bis (2,4-dinitrophenylhydrazone) derivative (15, 16) by a modified procedure developed at our Laboratory (17).

A 2,4-dinitrophenylhydrazine solution was prepared as follows: 0.5 g. of this reagent was dissolved in two ml. of concentrated sulfuric acid, and three ml. of water were added. After cooling, methanol was added to give a total volume

of 15 ml. The reagent was made up just prior to analysis and was not allowed to stand for any considerable length of time.

For the analysis, an aliquot of the solution to be analyzed, containing approximately five mg. of pure glutaraldehyde, was weighed into a 25 x 120 mm. centrifuge tube, and ten ml. of methanol were added. Then five ml. of the reagent prepared as described above were added, and the mixture allowed to stand at room temperature for one hour with occasional stirring. Approximately 0.5 g. of analytical grade Celite‡ was added, and the precipitated glutaraldehyde bis-(2,4-dinitrophenylhydrazone) was filtered off onto a fritted-glass funnel with suction. The centrifuge tube and precipitate were washed three times with five ml. portions of methanol. The filtrate was discarded and the receiver replaced with a clean, dry, 250 ml. filter flask. The centrifuge tube and the funnel were treated repeatedly (five 10-ml. portions) with hot ethylene dichloride until all of the bis(2,4-dinitrophenylhydrazone) was dissolved and collected in the receiving flask. Manipulations with methanol and ethylene dichloride were carried out in a hood to minimize exposure to these vapors. The ethylene dichloride solution of glutaraldehyde bis(2,4-dinitrophenylhydrazone) was transferred to a 100 ml. volumetric flask, cooled to room temperature, and made up to the mark with additional ethylene dichloride. For analysis, a one ml. aliquot of this solution was made up to volume in a 50 ml. volumetric flask with ethylene dichloride and the absorbance measured in a Beckman Model DB Spectrophotometer at a wavelength of 360 mμ (16) against a blank. The absorbance obtained is compared with that from a standard curve to give the glutaraldehyde content. Data on the aldehyde content of the spent tanning solutions are summarized in Tables I, II and III.

By choosing a larger sample the glutaraldehyde can be determined gravimetrically as the bis(2,4-dinitrophenylhydrazone) rather than spectrophotometrically.

Analysis of Glutaraldehyde-Tanned Leathers.—The entire amount of dried leather obtained from the miniature tanning experiments described above was cut into small squares (about ¼ inch), placed into a 125 ml. Erlenmeyer flask, covered with 15 ml. of 2*N* HCl and hydrolyzed by refluxing for two hours. The hydrolyzate was evaporated to dryness on a steam bath to remove excess HCl. The residue was then made up to the mark in a 50 ml. volumetric flask with distilled water and filtered through paper to remove the slight amount of insoluble matter. Approximately 0.50 g. of analytical grade Celite was added to facilitate filtration. The filtered hydrolyzate was diluted 50 fold with water before analyzing with the Cary Model 14 recording spectrophotometer, using a one cm. absorption cell. The ultraviolet absorption spectra for hydrolyzates of

UPTAKE OF GLUTARALDEHYDE BY PICKLED CABRETTA SKIN AT pH 4*

Experiment No.	Weight of Tanning System			Aldehyde Content of Spent Liquor		Initial Aldehyde	Aldehyde Consumed		Absorbance‡‡ at 265 mμ	
	Total† g.	Dried Leather‡ g.	Tanning Liquor** g.	mg./g.	Total, mg.	mg.	Total†† mg.	Normalized*** mg.	obs., Δ	Normalized***
1	10.53	1.57	8.96	0.573	5.14	50.27	45.13	29.0	0.25	0.16
2	10.60	1.58	9.02	1.543	13.90	75.41	61.51	39.0	0.33	0.21
3	10.58	1.62	8.96	2.963	26.52	100.07	73.55	45.0	0.43	0.27
4	10.50	1.60	8.90	4.980	44.33	125.68	81.35	51.0	0.41	0.26
5	10.53	1.57	8.96	7.900	70.80	150.10	79.30	50.0	0.47	0.30
6	10.59	1.56	9.03	11.690	105.60	200.13	94.53	61.0	0.53	0.40
7	10.66	1.57	9.09	14.990	136.30	250.17	113.87	72.0	0.57	0.36
8	10.52	1.48	9.04	21.820	197.30	304.71	107.41	73.0	0.54	0.36
9	10.62	1.58	9.04	29.730	268.70	406.28	137.58	87.0	0.63	0.40

*Sodium formate used as a buffering agent, 1:1 float.
†Total mass of skin, reagents and water.
‡Moisture-free basis.
**Determined by difference; i.e., total minus dried leather.
††Initial minus total in spent liquor.
‡‡Concentration of hydrolyzate for analysis was equivalent to the entire protein diluted to 2500 ml.
***Calculated to a basis of one gram of protein.

TABLE II

UPTAKE OF GLUTARALDEHYDE BY PICKLED CABRETTA SKIN AT pH 8*

Experiment No.	Weight of Tanning System			Aldehyde Content of Spent Liquor		Initial Aldehyde	Aldehyde Consumed		Absorbance‡‡ at 265 mμ	
	Total† g.	Dried Leather‡ g.	Tanning Liquor** g.	mg./g.	Total, mg.	mg.	Total†† mg.	Normalized*** mg.	obs., △	Normalized***
1	10.41	1.58	8.83	0.0353	0.31	50.27	49.96	32.0	0.30	0.19
2	10.48	1.65	8.83	0.0283	0.25	75.41	75.16	45.0	0.46	0.28
3	10.42	1.70	8.72	0.0287	0.25	100.07	99.82	59.0	0.58	0.34
4	10.54	1.70	8.84	0.2730	2.41	125.68	123.27	72.0	0.66	0.39
5	10.42	1.70	8.72	0.6420	5.60	150.10	144.50	85.0	0.79	0.46
6	10.71	1.73	8.98	2.8730	25.80	200.13	174.33	101.0	0.85	0.49
7	10.71	1.70	9.01	7.1200	64.10	250.17	186.07	110.0	0.88	0.52
8	10.66	1.75	8.91	13.4400	119.70	304.71	185.01	106.0	0.91	0.52
9	10.75	1.77	9.65	17.7200	170.96	406.28	236.19	133.0	0.94	0.53

*Sodium bicarbonate used as a buffering agent, float 1:1.
†Total mass of skin, reagents and water.
‡Moisture-free basis.
**Determined by difference; i.e., total minus dried leather.
††Initial minus total in spent liquor.
‡‡Concentration of hydrolyzate for analysis was equivalent to the entire protein diluted to 2500 ml.
***Calculated to a basis of one gram of protein.

TABLE III

UPTAKE OF GLUTARALDEHYDE BY CHROMED CABRETTA SKIN AT pH 4*

Experiment No.	Weight of Tanning System			Aldehyde Content of Spent Liquor		Initial Aldehyde	Aldehyde Consumed		Absorbance‡‡ at 265 mμ	
	Total† g.	Dried Leather‡ g.	Tanning Liquor** g.	mg./g.	Total, mg.	mg.	Total†† mg.	Normalized*** mg.	obs., △	Normalized***
1	10.67	1.38	9.29	0.762	7.07	50.07	43.00	31.0	0.25	0.18
2	10.64	1.34	9.30	2.458	22.86	75.10	52.24	39.0	0.31	0.23
3	10.64	1.40	9.20	4.080	37.54	100.14	62.60	45.0	0.36	0.26
4	10.64	1.38	9.26	8.283	76.47	150.20	73.73	53.0	0.41	0.30
5	10.73	1.45	9.28	11.280	104.66	200.28	95.62	66.0	0.52	0.36
6	10.72	1.45	9.27	20.300	188.12	300.40	112.28	77.0	0.59	0.41
7	10.76	1.45	9.31	31.200	290.47	400.50	110.03	76.0	0.62	0.43
8	10.80	1.44	9.36	40.580	379.79	500.70	120.91	84.0	0.65	0.45

*Experiments were run in triplicate and average values used.
Sodium formate used as buffering agent, float 1:1.
†Total mass of skin, reagents and water.
‡Moisture-free basis.
**Determined by difference; i.e., total minus dried leather.
††Initial minus total in spent liquor.
‡‡Concentration of hydrolyzate for analysis was equivalent to the entire protein diluted to 2500 ml.
***Calculated to a basis of one gram of protein.

glutaraldehyde-tanned collagen and for the blank (collagen) are shown in Figure 1. The absorption maximum at 265 mμ was characteristic for hydrolyzates of glutaraldehyde-tanned leathers, and absorbance at this wave-length was measured. The absorbance was corrected for the blank, and the data are shown in Tables I, II and III.

DISCUSSION

A previous communication from our Laboratory demonstrated the irreversible fixation of glutaraldehyde by collagen and the appearance of a characteristic absorption maximum at 265 mμ in the hydrolysate of the leather (14). The ultraviolet spectra of hydrolyzates of collagen and glutaraldehyde-tanned collagen are shown in Figure 1. Our previous studies on uptake of glutaraldehyde, as estimated indirectly by analysis of the tanning liquor (9), clearly showed increased uptake as pH of tanning increased. This is reflected in Figure 1 where the absorbance at 265 mμ, which is obtained by spectral analysis of the collagen hydrolyzate, also increased with increasing pH of tanning. Thus the spectral data appeared to correlate with the data on analysis of the tanning liquors.

Because of this apparent relationship, a series of miniature tanning experiments, on a quantitative basis, was conducted to correlate the ultraviolet data with that obtained by analysis of the tanning liquor. From a material balance on glutaraldehyde, its loss from the tanning liquor was obtained, and this was compared to the spectral data (absorbance at 265 mμ) obtained from analysis of the collagen phase. These data are presented in the last four columns of Tables I, II, and III. Because of the variability in the solids content of the pickled and chrome-tanned stock, the data were normalized by calculation to a unit basis; i.e., aldehyde consumed and absorbance were calculated to a basis of 1 g. of protein. The correlation of absorbance at 265 mμ with total glutaraldehyde consumed is plainly evident.

This correlation is more clearly seen in Figure 2 in which absorbance at 265 mμ (concentration approximately 0.6 mg. protein per ml.) was plotted against total glutaraldehyde uptake; i.e., aldehyde consumed (mg./g.) as calculated by difference. Although some scattering of points exists, especially above an uptake of about 100 mg./g. of protein, a reasonably good straight line can be drawn for these data. The data obtained with the chrome-tanned cabretta seemed to show the best fit to the line; and, furthermore, chrome did not interfere with spectral analysis in this region. In spite of the high variability of animal hides and skins, it is felt that this preliminary study does demonstrate that bound glutaraldehyde can be estimated by spectral analysis of the hydrolyzate of the leather. Since the abscissa (glutaraldehyde uptake) is expressed in terms of mg. per g. of tanned protein, the fixation, in terms of percent, can readily be calculated. The curve in Figure 2 appears to be useful only to an uptake of about ten percent

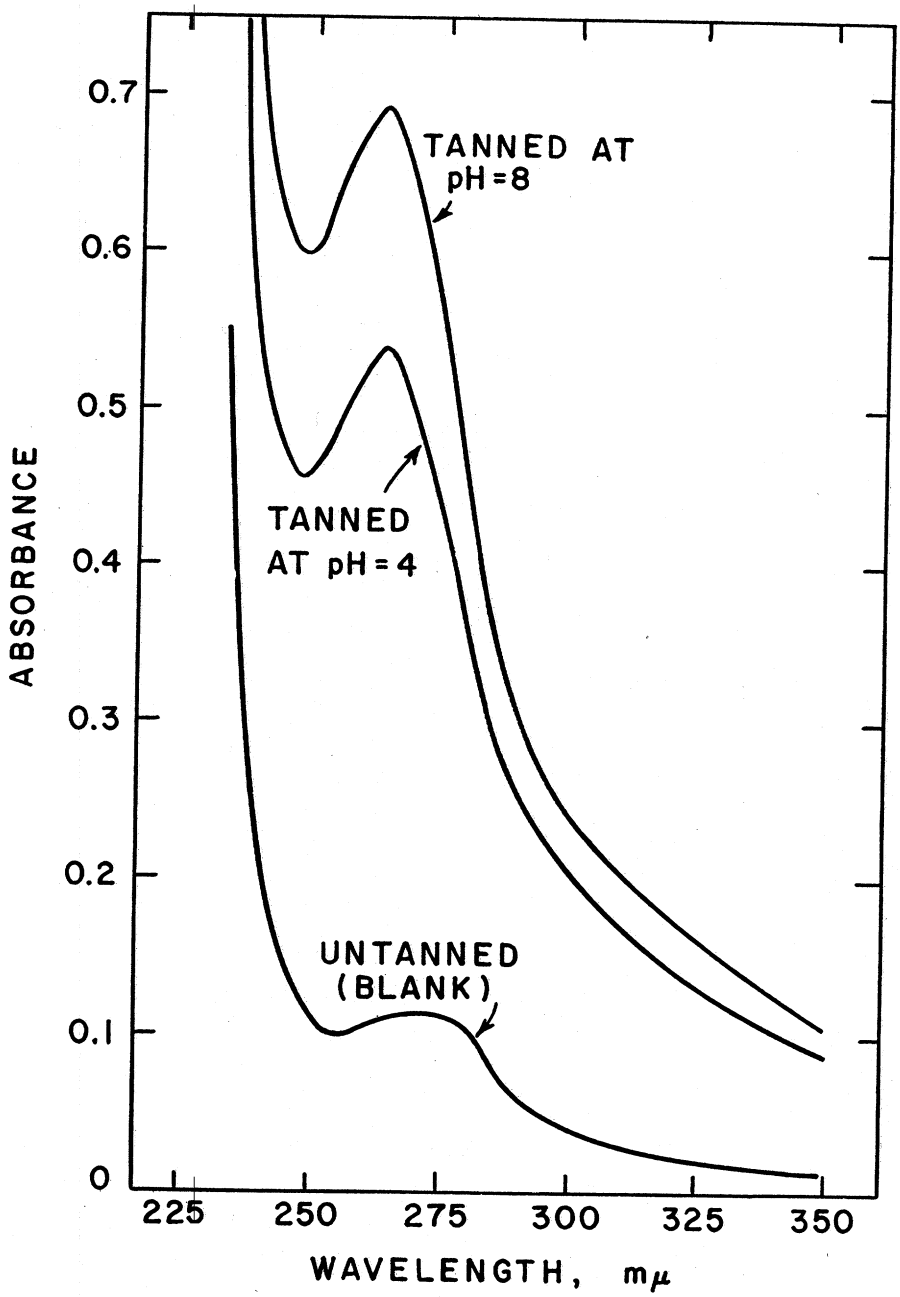

FIGURE 1.—Ultraviolet absorption spectra for glutaraldehyde-tanned cabretta. Hydrolyzate from 2.0 g. protein diluted to concentration of 0.8 mg. per ml.

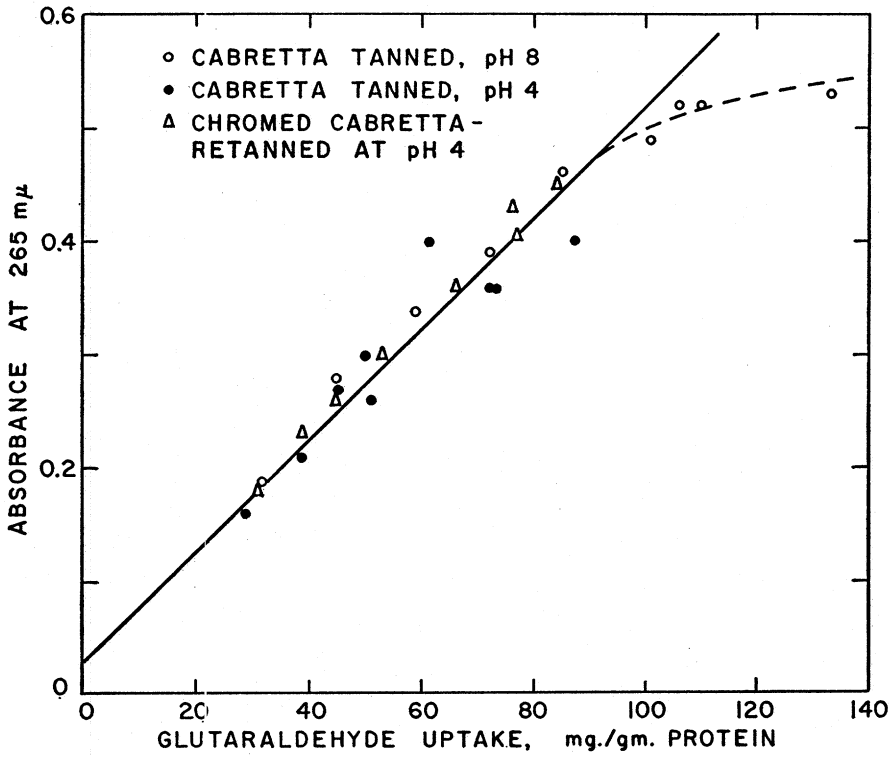

FIGURE 2.—Correlation of absorbance at 265 mμ with glutaraldehyde uptake.

(100 mg./g. protein). Current studies are underway to refine the spectral method and to demonstrate the nature of the products responsible for the absorption characteristic.

From these preliminary data the equation for the straight line drawn through the experimental points is as follows:

$$A = 0.025 + 0.00494X$$

Where

A = Absorbance observed from hydrolyzate of 1 g. protein diluted to 2500 ml.

X = Uptake of glutaraldehyde in mg. per g. tanned protein

Although chrome did not interfere with the ultraviolet spectral analysis, it appeared quite likely that other common materials used in finishing leather; for example, vegetable tannins, syntans, dyes, resins, finishes, etc., may present some obstacles. To determine if such may be the case, glutaraldehyde-tanned cabretta skins were retanned with ten percent their weight of sulfited quebracho,

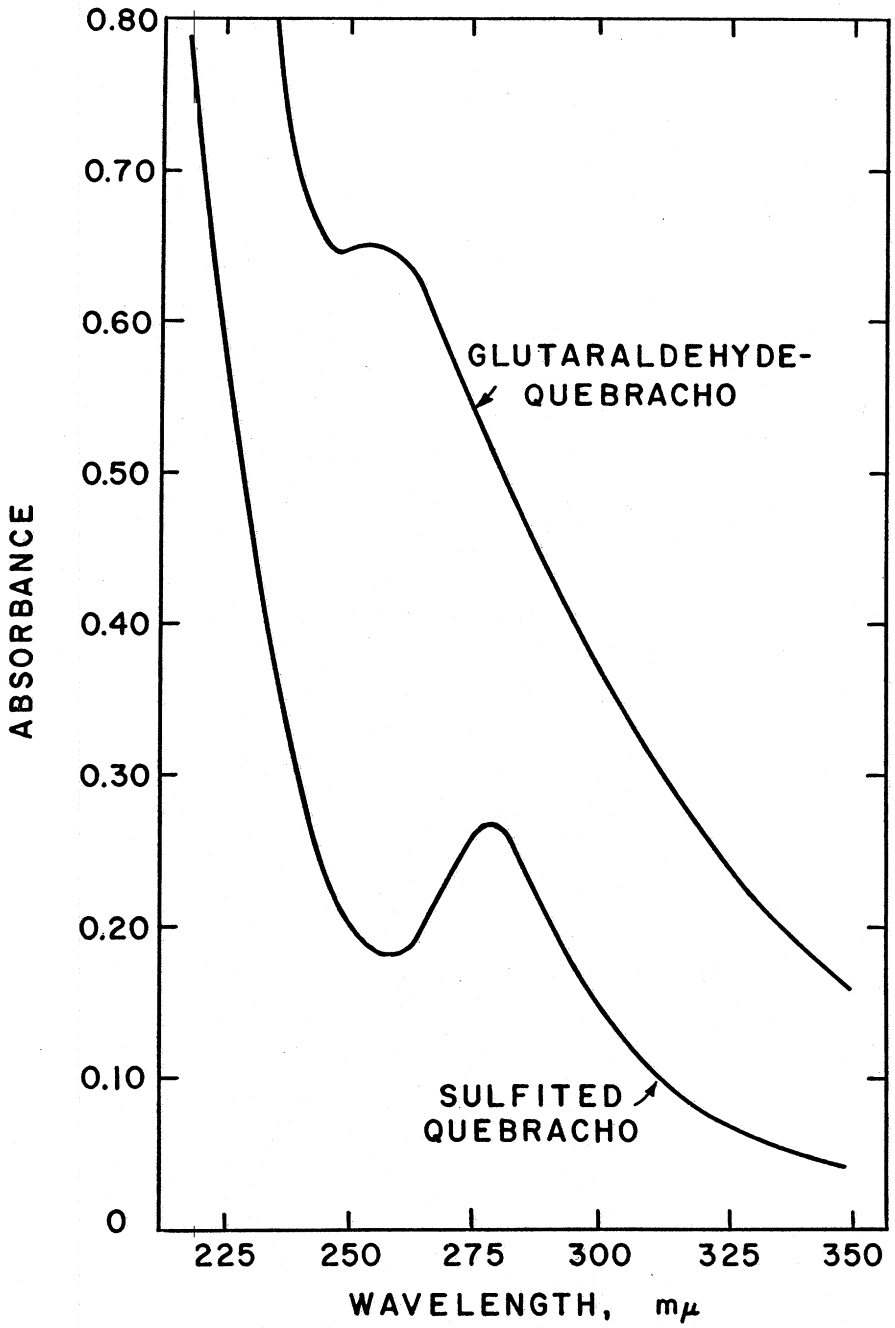

FIGURE 3.—Absorption spectra of glutaraldehyde-tanned leather retanned with sulfited quebracho.

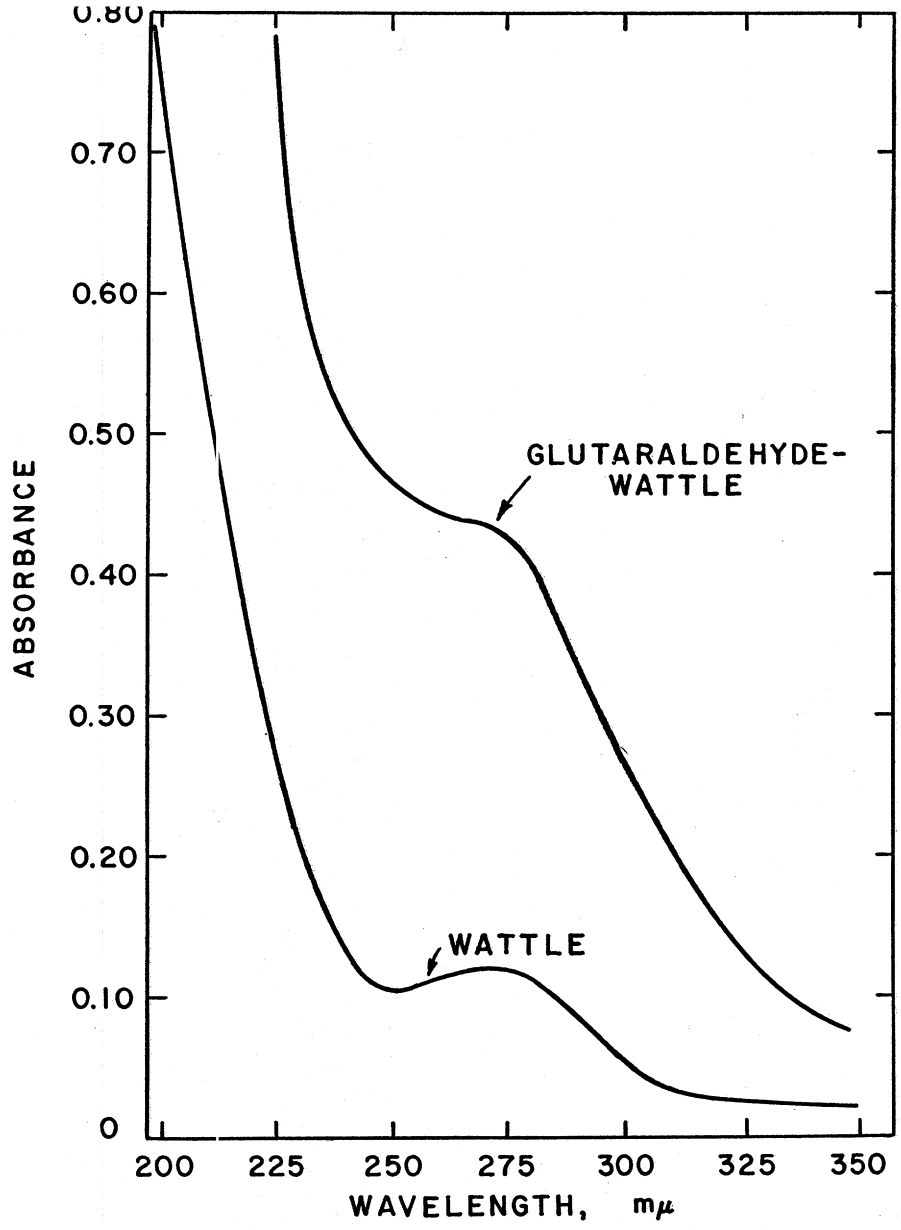

FIGURE 4.—Absorption spectra of glutaraldehyde-tanned leather retanned with wattle.

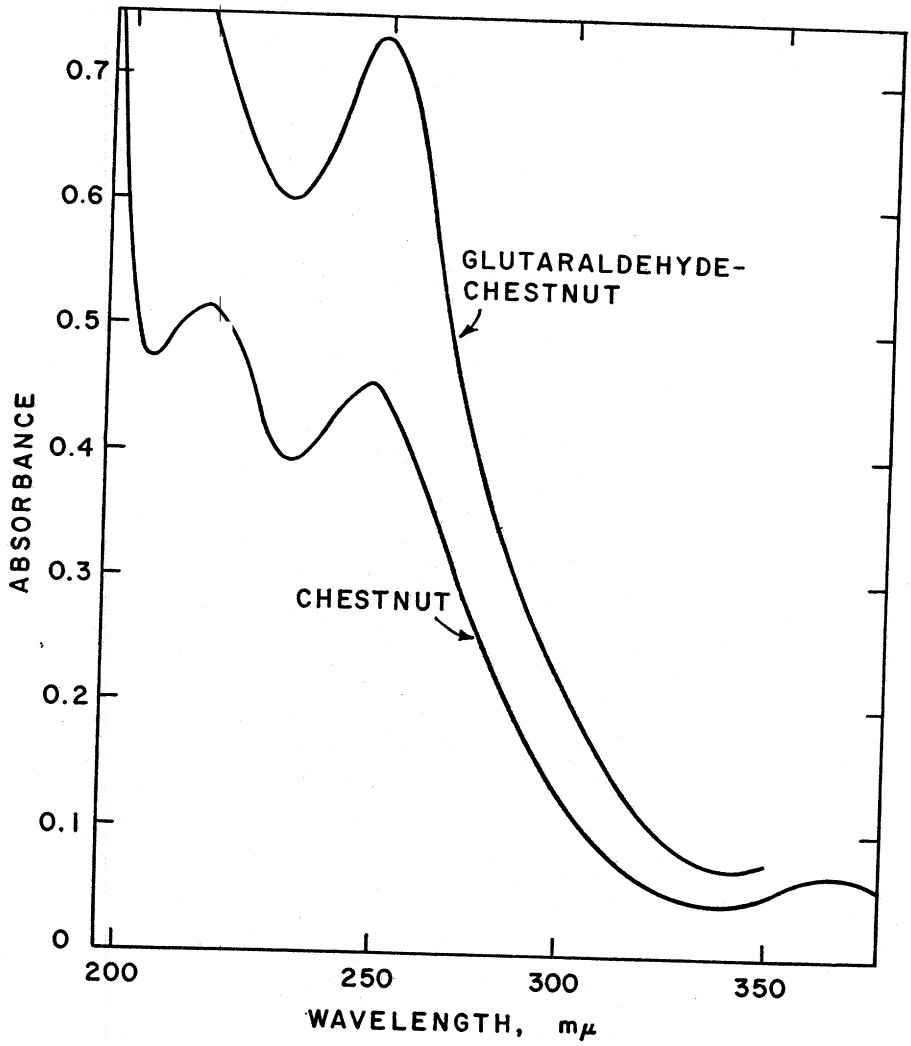

FIGURE 5.—Absorption spectra of glutaraldehyde-tanned leather retanned with chestnut.

wattle and chestnut. Two grams of the leathers were hydrolyzed by refluxing with 15 ml. of 6*N* HCl for eight hours. The absorption curves for these hydrolyzates are shown in Figures 3, 4, and 5. The vegetable tannin, itself, carried through the same hydrolysis procedure, served as a blank. It is quite evident that the natural tannins show a maximum absorption in this region, as has been demonstrated by previous investigators (18, 19). Hydrolysis of the vegetable-retanned glutaraldehyde leather resulted in definite changes in the ultraviolet absorption pattern. In the case of wattle and sulfited quebracho, especially the

former, the curve was characterized by a shoulder instead of a peak. In the case of chestnut, a sharp peak at 250 mμ, characteristically similar to that obtained from hydrolysis of the extract alone, was observed. Perhaps this difference in behavior can be attributed to the fact that chestnut is a hydrolyzable tannin, whereas wattle and quebracho are of the condensed type. Thus, vegetable tannins can be expected to complicate and interfere with the spectral analysis of bound glutaraldehyde. Curiously enough, these tannins also interfere in the determination of bound formaldehyde in its leathers. It seems, therefore, that aromatic materials used in leather processing are likely to interfere with spectral analysis for bound glutaraldehyde, and further study is needed to develop reliable procedures for analysis of finished leathers. Despite this limitation, the ultraviolet absorption analysis offers considerable promise as a tool for research studies in defining and elucidating the interaction between glutaraldehyde and collagen and also for analysis of glutaraldehyde-tanned leathers in which interfering chromophores are absent.

SUMMARY

A method for the direct determination of glutaraldehyde bound by collagen has been developed. This method is based on the novel observation of a characteristic absorption maximum at 265 mμ in hydrolyzates of glutaraldehyde-tanned collagen. The absorbance was shown to correlate with uptake of glutaraldehyde, as measured by loss of aldehyde from the tanning liquor. Chrome does not interfere with this spectral analysis; however, vegetable tannins, which contain chromophores absorbing strongly in the ultraviolet regions, do interfere. Interference can be expected from any aromatic type materials that are used in processing of leather. Examples of such materials include vegetable tannins, syntans, resins, dyes, and certain finishes.

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DISCUSSION

CHAIRMAN LOLLAR: Because of illness at the last minute, there will be a substitution in the discussion leader. Bob Aldrich of Union Carbide has volunteered to discuss this paper.

MR. ROBERT H. ALDRICH (Union Carbide Corporation): Mr. Korn, Dr. Lollar, Ladies and Gentlemen: Mr. Korn's work is significant for two reasons. First, it provides us all with a practical, easy and rapid method for analyzing glutaraldehyde pickup by analysis of the tanning liquors. This can be used by the tanner to optimize his operating conditions when first establishing a glutaraldehyde process. Later on, the method can be used as a convenient process-control tool.

The second -- and I think the most significant aspect of this paper — is that for the first time we have a method for analyzing glutaraldehyde quantitatively contained in the tanned leather. Before this, we could only analyze for contained glutaraldehyde or assay by a carbon 14 analysis. We could qualitatively determine glutaraldehyde using physical differences we measured in the leather with

glutaraldehyde vs. without glutaraldehyde. Being able to analyze the glutaraldehyde content in the leather is going to open new avenues of research for us and for other laboratories.

In particular, we will be able to study the distribution of glutaraldehyde throughout the leather and across the total area of the leather sample. We will be able to study the kinetics of glutaraldehyde tanning, both theoretically and practically.

We have noticed that under different tanning conditions, we obtain different surface characteristics. We haven't been able to analyze these effects and their relationship to "fixed" glutaraldehyde. With this new method, we will be able to tell how much glutaraldehyde is present in each location. It gives us a real tool with which to work.

The first question I'd like to ask, Mr. Korn, is: What do you hypothesize to be the chemical nature of that particular compound you hydrolyzed out of the leather? And, will there be a forthcoming method of analysis that will be more practical to the normal tannery's research laboratory in terms of equipment?

MR. KORN: We are pretty well convinced that glutaraldehyde is bound irreversibly to the collagen molecule, and when you hydrolyze this molecule, you break it at the peptide linkage but probably not at the glutaraldehyde binding site which may form a heterocyclic ring structure. This is speculative and we contemplate further research to establish the nature of the material absorbing in this region.

MR. ALDRICH: Do you think you will ever be able to analyze with other means than ultraviolet spectrum?

MR. KORN: For the analysis of tanning liquors, spectral analysis is not necessary and can be accomplished very satisfactorily by a gravimetric procedure. For analysis of the hydrolyzate, the spectral method is the only analytical means available so far. We hope that further work may contribute to new approaches to this problem.

MR. ALDRICH: Are there any questions from the floor? I have one more question myself. Getting a copy of the paper prior to this discussion, we had a chance to work with it ourselves in our own laboratory. We analyzed some finished leather that we knew contained glutaraldehyde but we weren't quite sure how much. We got some excellent results on undyed and unfinished leathers where we could readily see the percentage glutaraldehyde that showed up according to your curves. On vegetable-tanned leather, highly oiled leathers, and black leathers, we had some difficulty in using this method. On white leather, the curve was spotty and it was very difficult to determine the exact amount.

Do you think there is a way of masking out these interferences, or another way of preparing finished leather, so that it could be analyzed by your method? Or, do you think we should measure it just directly out of the tanning drum?

MR. KORN: We have done a lot of work on finished leathers that we knew contained glutaraldehyde, but our success has been only partial. We have found that more drastic hydrolysis (8 hours with 6*N* HCl) was helpful. But there were some finished leathers that gave us trouble. We obtained good results with stock retanned with glutaraldehyde, in the blue, and also with finished white leathers. Brown and black leathers gave inconclusive results. Part of the trouble can be accounted for by the fact that aromatic substances such as syntans, dyes, vegetable extracts, finishes, etc. will interfere with the spectral analysis. We are continuing our studies to overcome this difficulty.

MR. ALDRICH: Any further questions? I'd like to thank Al and his associates for a good piece of work here.

CHAIRMAN LOLLAR: Thank you, Bob and Al.